ORGANIC AND BIOLOGICAL CHEMISTRY

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Spectral Studies of Denatured Phycoerythrins[1]

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RECEIVED APRIL 1, 1960

Absorption spectral changes accompanying the fluorescence-quenching denaturation of phycoerythrins in acid and urea solutions have been studied. Derivatives of the denatured chromoproteins have been prepared and the results interpreted in terms of chromophore–protein attachment. Evidence for the presence of two different bile pigment chromophores in R-phycoerythrin is presented.

Native and denatured proteins differ in their ultraviolet absorption spectra.[2] For example, ribonuclease, which contains tyrosyl groups, absorbs more intensely and at a wave length longer by 2.5 mμ than the denatured protein or free tyrosine. The absorption spectra of extraneous chromophores attached to proteins are also perturbed on denaturation of the proteins.[3,4] Such spectral shifts have been variously interpreted as resulting from the cleavage of hydrogen and possibly hydrophobic bonds,[2] variations in electronic polarizability in the environment of the light-absorbing group,[3] and disruption of "frozen" water envelopes surrounding areas of protein surface.[4]

We have studied the changes in chromophore absorption spectra resulting from the denaturation of phycoerythrins, a group of fluorescent algal chromoproteins.[5] These chromophores are strongly attached tetrapyrrolic bile pigments, whose structures have not yet been finally established.[6,7]

Lemberg[8] showed that dilute acid quenches the fluorescence and alters the absorption spectrum of R-phycoerythrin. All the phycoerythrins we have examined are denatured, with loss of fluorescence, by acid and more slowly by urea. Increased chromophore reactivity is associated with denaturation.

Experimental

Preparation of Phycoerythrins.—R-Phycoerythrin was obtained from *Ceramium rubrum* and B-phycoerythrin from *Rhodochorton floridulum*. Both algae were collected on the shore near Galway. *Hemiselmis rufescens*, the source of cryptomonad phycoerythrin, was cultured in white fluorescent light. The phycoerythrins were released from the algae into distilled water and purified by fractional precipitation with ammonium sulfate and/or chromatography on tricalcium phosphate–Supercel.[9]

Instrumentation.—Absorption spectra were determined with a Unicam S.P. 500 spectrophotometer. The absorption peaks of denatured phycoerythrins are, in general, rather broad and, as a result, difficult to locate accurately. To facilitate discussion an λ_{max} is assigned to each peak, but the accuracy and reproducibility of these values was ±1 mμ.

Fluorescence spectra were determined with the fluorospectrophotometer described by Olson and Amez,[10] using an RCA IP 22 red-sensitive photomultiplier. At the exciting wave lengths of 490 and 540 mμ the emitted radiation was passed through Schott filters GG 14 and OG 2, respectively. *p*H values were obtained with a Beckman 9600 Zeromatic *p*H meter.

Results

Effects of Dilute Acid.—Acidification to *p*H 3 of aqueous solutions of phycoerythrins brings about immediate reversible quenching of their fluorescence; irreversible quenching occurs on long standing, or in more concentrated acid.

While its O.D. is decreased, the λ_{max} of cryptomonad phycoerythrin is not appreciably shifted in acid (Fig. 1). A more striking perturbation leads to a similar spectrum (λ_{max} 557 mμ) on acidification of B-phycoerythrin (λ_{max} 544 and 566 mμ in neutral aqueous solution). Under the same conditions, the two long wave length peaks of native R-phycoerythrin are also replaced by a single maximum at 557 mμ (Fig. 2). The position of the 497 mμ peak of this chromoprotein is unaffected even by concentrated hydrochloric acid. However, as was noted by Lemberg,[8] the extinction at this wave length relative to that at 557 mμ increases when R-phycoerythrin is allowed to stand in acid.

Effects of 8 *M* Urea.—The fluorescence of phycoerythrins is practically completely quenched on standing in 8 *M* urea solution for 24 hours at 3–5°. (The related phycocyanins lose their fluorescence immediately in 8 *M* urea.)

Urea shifts the absorption maximum of cryptomonad phycoerythrin by about 15 mμ to shorter wave lengths (Fig. 1) and B-phycoerythrin in urea solution possesses a similar single-peaked absorption spectrum (λ_{max} 540 mμ). While the 497 mμ maximum of R-phycoerythrin is not shifted in urea solution, the two long wave length maxima of the native chromoprotein are replaced by a single maximum at 540 mμ (Fig. 2).

The spectra of urea and acid-denatured phycoerythrins are *p*H dependent and, to judge from spectra, they are interconvertible.

Zinc Complex Salt Formation by Denatured Phycoerythrins.—All phycoerythrins yield a chloroform-soluble chromophore (phycoerythrobilin) when hydrolyzed with concentrated acid.[7,11] This pigment is non-fluorescent, but it forms an orange-fluorescing complex salt with zinc acetate and also fluoresces brilliantly in the native chromoproteins. Urea-denatured, non-fluorescent phycoerythrins also form fluorescent zinc complexes; however, their fluorescence is weaker and redder than that of native phycoerythrins (see Table I).

The absorption maximum of the denatured cryptomonad phycoerythrin-zinc complex lies at 586 mμ, with a smaller peak at 542 mμ (Fig. 1). The zinc complex salt of denatured B-phycoerythrin possesses a similar spectrum. Absorption and fluorescence spectra (Table I) indicate that zinc

(1) This investigation was supported by the U. S. Army European Research Office (Contract DA-91-591-EUC-1071).

(2) W. Kauzmann, *Advances in Protein Chem.*, **14**, 1 (1959).

(3) E. J. Williams and J. F. Foster, *J. Am. Chem. Soc.*, **82**, 242 (1960).

(4) I. M. Klotz, *Science*, **128**, 815 (1958).

(5) The three phycoerythrins used in this study may be distinguished by their visible absorption maxima in neutral aqueous solution: cryptomonad phycoerythrin (556 mμ), B-phycoerythrin (544 and 566 mμ), and R-phycoerythrin (497, 542 and 568 mμ).

(6) R. Lemberg and J. W. Legge, "Hematin Compounds and Bile Pigments," Interscience Publishers, Inc., New York, N. Y., 1949.

(7) C. Ó hEocha, *Arch. Biochem. Biophys.*, **73**, 207; **74**, 493 (1958).

(8) R. Lemberg, *Ann.*, **477**, 195 (1930).

(9) F. T. Haxo, C. Ó hEocha and P. Norris, *Arch. Biochem. Biophys.*, **54**, 162 (1955).

(10) J. M. Olson and J. Amez, *Biochim. Biophys. Acta*, **37**, 14 (1960).

(11) C. Ó hEocha, in "Comparative Biochemistry in Photoreactive Systems," M. B. Allen ed., Academic Press, Inc., New York, N. Y., 1960, p. 181.

TABLE I

ABSORPTION AND FLUORESCENCE MAXIMA OF CRYPTOMONAD PHYCOERYTHRIN AND DERIVATIVES

Compound	Absorption maxima, mμ	Approximate fluorescence maxima, mμ[a]
Phycoerythrobilin	576 (acid chloroform)	..
Phycoerythrobilin-zinc complex	583 (chloroform)	590
Native cryptomonad phycoerythrin	556 (buffer, *p*H 6.5)	580
Denatured cryptomonad phycoerythrin	540 (8 *M* urea)	..
Denatured cryptomonad phycoerythrin-zinc complex	542; 586 (8 *M* urea)	600

[a] Exciting radiation of wave length 540 mμ was used.

ions form a complex salt with phycoerythrobilin groups on these denatured proteins.

The spectrum of native B-phycoerythrin is not altered by the addition of zinc acetate; if a complex were formed with zinc, an absorption maximum would be expected at 583–586 mμ. The native protein must mask the zinc complex-forming sites of the chromophore units.

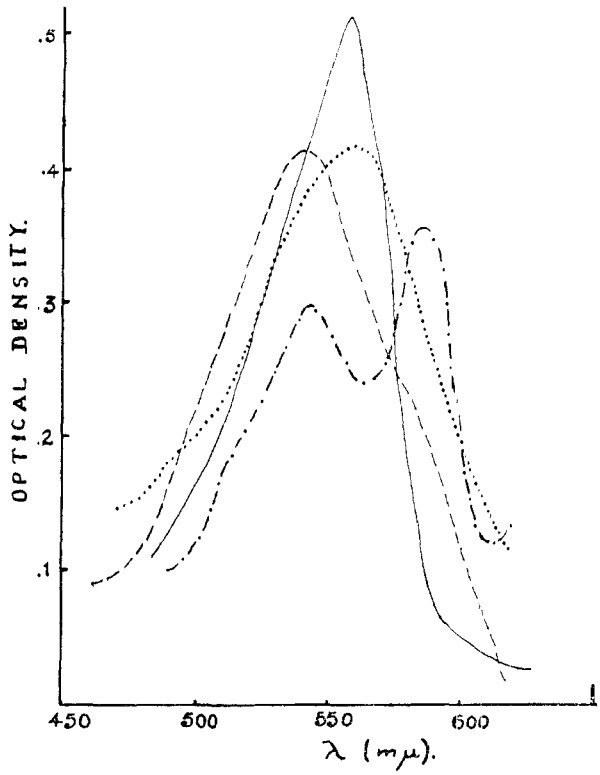

Fig. 1.—Absorption spectra of cryptomonad phycoerythrin solutions: ———, at *p*H 6.5; ....., at *p*H 1; – – – –, in 8 *M* urea for 24 hours; –·–·–, in 8 *M* urea + zinc acetate.

The addition of zinc acetate to denatured R-phycoerythrin produces a new maximum at 586 mμ, while the 497 mμ maximum is shifted to 512 mμ. This latter shift also occurs when zinc acetate is added to *native* R-phycoerythrin, the long wave length peaks of which, like those of native B-phycoerythrin, are unaffected by this reagent (Fig. 2).

Discussion

Phycoerythrobilin, a chromophore of all three phycoerythrins, is attached to the proteins by covalent as well as labile bonds and, according to Lemberg, it is the latter which are broken by denaturing agents, with consequent loss of chromoprotein fluorescence.[6,8] In view of the many possible effects of urea and acid on the secondary structures of proteins,[2–4] any interpretation of our results in terms of the labile chromophore–protein bonds must be tentative.

There is evidence that an imino group of the flavin mononucleotide chromophore of the old yellow enzyme is hydrogen bonded to a tyrosyl group of the protein,[12] and the pyrrole nitrogens of phycoerythrobilin may be capable of similar bonding. Intermolecular hydrogen bonding probably occurs in pyrrole and related compounds,[13] while intramolecular hydrogen bonds have been reported in the tetrapyrroles porphin and chlorin.[14]

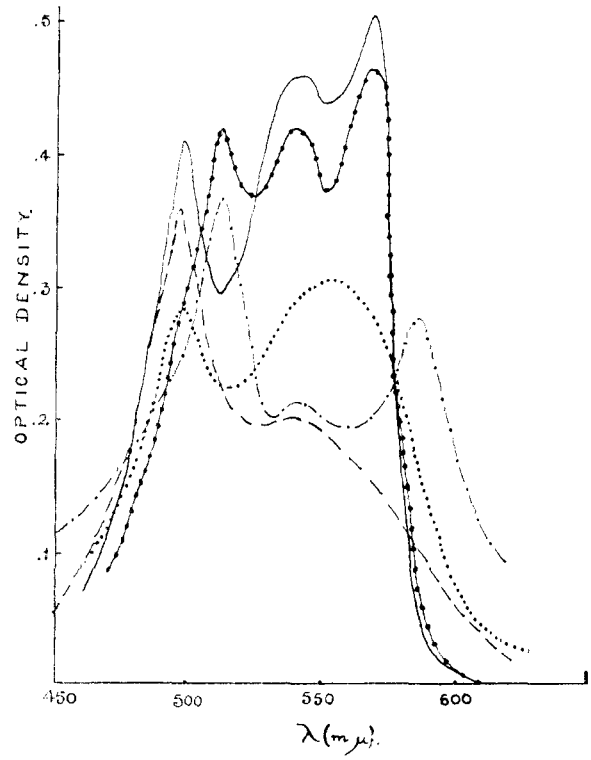

Fig. 2.—Absorption spectra of R-phycoerythrin solutions: ———, at *p*H 6.5; ....., at *p*H 1; •—•—•—•, at *p*H 7.0 + zinc acetate; – – – –, in 8 *M* urea for 24 hours; –·–·–, in 8 *M* urea + zinc acetate.

Analyses indicate that the bile pigments of the algal chromoproteins, unlike most tetrapyrrolic chromophores, are naturally uncomplexed with metals.[6,15] Zinc complex salt formation by bile pigments occurs through their nitrogen atoms.[16] The phycoerythrobilin chromophores of denatured phycoerythrins form zinc complexes readily, while

(12) H. Theorell, "IVth International Congress of Biochemistry," Vol. VIII, Pergamon Press, London, 1959, p. 168; K. Yagi, *ibid.*, p. 175.

(13) L. J. Bellamy, "The Infra-red Spectra of Complex Molecules," Methuen, London, 1958.

(14) S. F. Mason, *J. Chem. Soc.*, 976 (1958).

(15) A. Hattori and Y. Fujita, *J. Biochem.* (*Japan*), **46**, 633 (1959).

(16) C. H. Gray, "The Bile Pigments," Methuen, London, 1953.

those of native phycoerythrins do not react at all. The native proteins mask the pyrrole nitrogens of the phycoerythrobilins, possibly through direct hydrogen bonding. By breaking these bonds, urea quenches the fluorescence of the chromophores and exposes their pyrrole nitrogens to zinc ions.

The spectra of cryptomonad and B-phycoerythrins, which differ greatly in neutral aqueous solution,[5] are similar in acid and 8 *M* urea solutions (λ_{max} at 557 and 540 mμ, respectively). Phycoerythrobilin is the only pigment obtained from these two chromoproteins, but there are probably many phycoerythrobilin units per protein molecule.[6] The single absorption maximum of cryptomonad phycoerythrin indicates that its phycoerythrobilin–protein attachments are of one kind, while the two-peaked spectrum of native B-phycoerythrin indicates that some of its phycoerythrobilins are bound differently from the rest, depending, presumably, on the neighboring protein environment. The 544 mμ peak of native B-phycoerythrin corresponds to that of the urea-denatured chromoprotein and may be attributed to non-hydrogen bonded, non-fluorescent chromophores, the 566 mμ maximum being due to hydrogen bonded, fluorescent chromophores.

At wave lengths longer than 530 mμ, the spectrum of R-phycoerythrin, whether in water, acid or urea solutions, is similar to that of B-phycoerythrin and, therefore, we consider that its 568 and 542 mμ maxima are attributable to hydrogen bonded and non-hydrogen bonded phycoerythrobilins, respectively. The 497 mμ maximum of R-phycoerythrin, unlike those at 542 and 568 mμ, is not shifted on denaturation, but is shifted when zinc acetate is added to the native protein (Fig. 2), and, since it behaves independently of the phycoerythrobilin maxima, it must be attributed to a different pigment. These results also indicate that the pyrrole nitrogens of the 497 mμ chromophore are not masked by hydrogen bonding with the native protein.

Hydrolysis studies indicate that the 497 mμ chromophore is a urobilinoid pigment.[11] Both isomerization and oxidation of phycoerythrobilin yield such pigments, whose zinc complexes correspond in their absorption maxima to the 497 mμ chromophore-zinc-protein maximum at 512 mμ. That there are two independent chromophores on R-phycoerythrin is also indicated by the fluorescence spectra of the zinc complex of the denatured chromoprotein, which displayed a maximum at 520 mμ when excited at $\lambda_{490\ m\mu}$, and at 600 mμ when excited at $\lambda_{540\ m\mu}$.

Acknowledgments.—We wish to thank Dr. L. N. M. Duysens, Leiden, for permitting P. O C. to determine fluorescence spectra in his laboratory; Mr. J. Amez for invaluable help in making the fluorescence spectral measurements; Dr. Mary Parke, Plymouth, for the *Hemiselmis* culture, and Miss D. Curley who cultured the *Hemiselmis* and who assisted in the purification of the phycoerythrins.

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Requirements for Stereospecificity in Hydrolysis by α-Chymotrypsin. Diethyl β-Acetamidoglutarate[1]

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RECEIVED JUNE 30, 1960

Diethyl β-acetamidoglutarate has been prepared and hydrolyzed by α-chymotrypsin. Lacking the structural features of typical substrates for this enzyme, it is hydrolyzed slowly but asymmetrically, leading to (+)-ethyl hydrogen-β-acetamidoglutarate, $[\alpha]^{25}D$ + 5.9°, which has also been prepared by resolution of the *dl* compound. Experiments with inhibitors indicate that this hydrolysis, and the similar asymmetric hydrolysis of diethyl-α-acetamidomalonate, are caused by the one active site of the enzyme. Association of substrate with enzyme via the characteristic structural features, which when present may lead to high reactivity and chemical specificity, is not required for and may not be responsible for stereospecificity.

Introduction

Consideration of the specificity of enzymatic reactions indicates that it may be desirable to elucidate two separate, but perhaps not entirely independent, sets of factors: first, those elements of structure in the substrate which contribute to high reactivity and are responsible for chemical specificity, and, second, those structural features which, by diastereomeric or conformational interactions with groups on the enzyme, lead to stereospecificity.[2] This may imply that high reactivity and stereospecificity are determined neither at identical sites on the enzyme nor at the same time. However, the stereospecificity of an enzyme frequently is shown by its causing one enantiomorph of a pair to undergo reaction very rapidly and the other very slowly or not at all and is thus intimately related to measurement of chemical reactivity. Thus a distinction may fail to be made between the two sets of factors and they may be thought to be one and the same, the requirements for stereospecificity, in effect, not being subjected to independent investigation.

Attention was especially centered on enzyme stereospecificity some years ago when it was found that symmetric molecules of type Ca,b,d,d could be formed, enzymatically, asymmetrically labelled and could undergo enzymatic reaction asymmetrically at the two sites *d*.[3] The explanation of this in terms of three point contact[4] was plausible,

(1) (a) We are pleased to acknowledge support of this work by the Division of Research Grants, The National Institutes of Health, RG 4584. (b) For a preliminary report, see S. G. Cohen and E. Khedouri, *Nature*, **186**, 75 (1960).

(2) S. G. Cohen and L. H. Klee, THIS JOURNAL, **82**, 6038 (1960).

(3) For references on this subject, see ref. 2.

(4) A. G. Ogston, *Nature*, **162**, 963 (1948).